**Synopsis**
Composite resin (CR) is used in vivo as a root perforation sealer and as a retrograde obturation material following apicoectomy, but it does not possess adequate biocompatibility. Effective biological modification of the CR surface is thus needed to improve its biocompatibility. Carbon nanotubes (CNTs) are a type of nanocarbon material formed from cylindrical graphene sheets with a diameter of about 10 to 200 nm. CNTs have garnered attention for useful properties such as promoting cell adhesion and proliferation. The present study evaluated cell adhesion and proliferation on CR discs coated with CNTs. CR discs were coated by immersion in a dispersion of CNTs (Nanocyl NC7000, 9.5 nm diameter). The CNT coating was then observed with a scanning electron microscope (SEM) to investigate its surface properties. The effects of CNT coating were also investigated by conducting cell culture assay using osteoblasts. The coating process enabled CNTs to adhere to the resin surface, resulting in good adhesion and spreading of the osteoblasts that indicated a high level of cell proliferation. The study findings showed that coating the surface of CRs with CNTs improved their biocompatibility.

**Key words:** Carbon nanotubes, Composite Resin, Cell proliferation, Biocompatibility

**Introduction**
Composite resins (CR) have been used in the field of dentistry for fillings and abutments, and as root perforation sealers and retrograde obturation material following apicoectomy [1-5]. In vivo dental materials must possess biocompatibility and biostability, as well as being easy to handle. The ability to induce and regenerate lost periodontal tissue on the CR surface could enable better prediction of treatment outcomes. However, CR don’t have superior biocompati-
bility and there are a few reports of successful periodontal tissue regeneration in vivo [6,7].

Meanwhile, advances in nanotechnology have given rise to a considerable amount of literature on technologies and applications of biomaterial surfaces modified with nanocarbon materials such as carbon nanotubes (CNTs) and graphene [8-10]. CNTs in particular have been reported to exhibit various biological properties [11], such as promoting cell adhesion and proliferation [12] and protein absorption [13], and have garnered interest for their use as carriers in drug delivery systems [14], cell culture scaffolds and biomaterials such as artificial joints and bones [15]. In recent years, outstanding dispersion technologies have enabled biomaterials to be coated with CNTs. Hirata et al. described coating collagen sponges with CNTs and performing 3D cell culture, resulting in early osteoblast adhesion and proliferation deep into the sponges, followed by cell differentiation [16]. The authors also reported the effects of this CNT coating method on the proliferation of bone marrow-derived stromal cells by functionalizing CNTs with basic fibroblast growth factor (bFGF) prior to coating [17].

These study findings suggest that modifying CR surfaces with CNTs improves their biocompatibility by imbuing them with CNT properties and creating a suitable environment for cell proliferation. In the present study, the properties of CR surfaces were observed after coating in CNT dispersion, and their ability to promote cell proliferation was evaluated.

Materials and Methods

**CNT coating of CR surfaces**

A mold with a diameter and height of 3 mm × 1 mm was fabricated and filled with CR (Beautiful Flow Plus, UniFil Flow; A2, Shofu, Kyoto, Japan), which was then cured for 20 seconds using a cordless LED light (Pencure, Morita, Kyoto, Japan). The CR surface was polished using #600 waterproof sandpaper before ultrasonic cleaning for 5 minutes. Multi-wall CNTs (Nanocyl NC 7000, 9.5 nm diameter, Nanocyl, Sambreville, Belgium) were then dispersed in n-methylpyrrolidone containing 0.2 wt% sodium cholate as the dispersant and the concentration was adjusted to 0.5 wt% for use in the test. CR was coated by immersing it in the dispersant, and the resulting CNT-CR was used as the test sample. Coating times were set at 20, 60 and 180 seconds. A CR not coated with CNTs was used as the control. After removing the dispersant and solvent and dewatering in an alcohol series, Pd-Pt deposition was performed and the sample surface coating was observed using SEM (S-4000; Hitachi, Tokyo, Japan) at 10 KV.

**Cell morphology**

MC3T3-E1 mouse osteoblasts (1 × 10⁴; RIKEN BioResource Center, Tsukuba, Japan) were seeded onto the CNT-CR and CR and cultured under 5% CO₂ at 37°C. Culture used a medium (MEM alpha GlutaMAX™-I; Thermo Fisher Scientific, Waltham, MA) containing fetal bovine serum (FBS, Qualified; Thermo Fisher Scientific) and 1% antibiotic (penicillin/streptomycin; Thermo Fisher Scientific).

The CNT-CR (60-second coating) and CR samples cultured for 1 day and 3 days were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 30 minutes and were then rinsed with a cacodylate buffer solution. After dewatering and drying using an alcohol series and coating with Pd-Pt, the surface cells were observed using SEM (S-4000; Hitachi, Tokyo, Japan) at 10 KV.

Five sites with an area of 400 μm × 300 μm were selected for each sample and the cell count of each site was measured.

Statistical analyses of cell counts results were performed using t-test with a 5% significance level. A statistical procedure was performed using a software package (IBM SPSS 11.0; IBM SPSS Japan, Tokyo, Japan).

**Proliferation assay**

MC3T3-E1 cells (1 × 10⁵) were seeded onto the CNT-CR and CR and cultured under 5% CO₂ at 37°C. Culture used a medium (MEM alpha GlutaMAX™-I; Thermo Fisher Scientific, Waltham, MA) containing fetal bovine serum (FBS, Qualified; Thermo Fisher Scientific) and 1% antibiotic (penicillin/streptomycin; Thermo Fisher Scientific).

Cells cultured in a medium without the CNT-CR and CR sample are used as control. After culturing for 1, 3, 7 and 10 days, cell proliferation assay was performed using kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions, and the assay results were evaluated. Absorb-
ance was measured using a microplate reader at a wavelength of 450 nm. Statistical analyses of proliferation assay results were performed using one-way ANOVA and Tukey HSD test with a 5% significance level. A statistical procedure was performed using a software package (IBM SPSS 11.0; IBM SPSS Japan, Tokyo, Japan).

**Results**

CNT-CR had a brown coloration on macroscopic observation (Figure 1). In the CNT-CR with a coating time of 20 seconds, SEM observation showed that the CNTs had dispersed onto the CR (Figure 2-b) In the samples coated for 60 and 180 seconds, a thin CNT mesh structure was observed on both the resin matrix and filler (Figure 2-c and 2-d).

![Figure 1](image1.png)

**Figure 1**
Composite resin with CNT coating
(a) No coating (b) 20 seconds (c) 60 seconds (d) 180 seconds

![Figure 2](image2.png)

**Figure 2**
SEM images of CNT coating (bar = 1 μm)
(a) No coating (b) 20 seconds (c) 60 seconds (d) 180 seconds
In the cell culture assay, the CNT-CR samples exhibited abundant cells but the CR samples had a low cell count (Figure 3, 4). The CNT-CR samples also exhibited marked adhesion of filopodia to CNTs (Figure 5).

Cell counts showed that the number of cells...
adhering to the CNT-CR was twice that of the control at 1 day, and 3 times that of the control at 3 days. Statistical analysis also demonstrated a significant difference when compared with CR (Figure 6).

The test results indicated that the CNT-CR was significantly higher than the CR at Day 10 (Figure 7).

**Discussion**
To the best of our knowledge, there are no published studies on improving the biocompatibility of CR, which are mainly used in dental fillings and abutments for use as apical sealants in dental perforations and retrograde obturation by coating the CR surface with CNTs. Beside CR, various materials have been used as filling mate-

![Figure 6](image6.png)

**Figure 6**
Cell adhesion counts after 1 day and 3 days (mean cell count at 5 sites: 400 μm × 300 μm)

![Figure 7](image7.png)

**Figure 7**
Cell proliferation
Evaluation of in vivo cell proliferation was performed using CCK-8 assay.
Materials, including amalgam, reinforced zinc oxide eugenol cement and MTA cement [18, 19]. Amalgam is problematic because it contains mercury [20], while reinforced zinc oxide eugenol cement has poor biocompatibility and tooth bonding properties [21]. MTA cement reportedly has good sealing properties in perforation and root obturation sites and promotes the formation of hard tissue in vivo [22, 23], but takes a long time to harden completely. CR have some superior characters, such as bonding to dentin and sealing properties and easy handling, and can be cured in a short time by exposure to light, making it an effective option in procedures that may involve bleeding. There have also been reports of cementum-periodontal membrane regeneration around the CR, indicating that it has low biological toxicity [6]. If these existing surface properties could be further enhanced by applying a coating of CNTs, the biocompatibility of the CR surface could be dramatically improved through the addition of CNT properties, which might make it possible to induce regeneration of periodontal tissue on the CR.

In our experiment, SEM observation of the CR samples after immersion for 20 seconds showed that the CNTs has dispersed on the CR surface, while immersion for more than 60 seconds resulted in the formation of a thin CNT mesh structure over the entire CR surface. There were no differences in CNT structure at 60 and 180 seconds immersion. These findings suggest that immersion for 20 seconds is an insufficient coating time, and that immersion for 60 seconds or more enables sufficient CNT coating of the CR.

SEM observation revealed an abundance of cells on the CNT-CR, and the cell count results also demonstrated that the CNT-CR samples had significantly higher counts than the control samples. Moreover, evaluation of cell proliferation at 1, 3, 7 and 10 days showed that the CNT-CR samples possessed higher proliferation capacity. CNT networks give the cells a hydrophobic fibermesh environment that increases their surface area and coarseness, thus improving cell adhesion and proliferation [24]. SEM findings of the present study also showed that filopodia had adhered to the CNTs, suggesting that the cells could selectively use CNTs as a scaffold. Dish cultures of various cells coated with CNT membranes have also yielded good cell adhesion and spreading [14]. In addition, studies by Nishida and Hirata have described improved cell adhesion on Ti surfaces coated with CNTs, and Hirata et al. reported that 3D collagen sponges coated with CNTs showed improved initial cell adhesion inside the sponge [12, 16]. Similarly, in the present study, coating the CR surface with CNTs created a CNT mesh structure with a 3D morphology that improved the CR surface characteristics and cell proliferation. The study findings suggested that this CNT coating method can improve the surface compatibility of CRs used in dental materials.

There has been a considerable amount of research on the safety of CNTs when used in vivo [24-26]. In a study on CNT diameters, nanotubes ≥ 50 nm were reported to penetrate cells, and administration of nanotubes with diameters of ≥50 nm to rats caused severe inflammation of the abdominal cavity [27]. Nishida et al. reported that CNTs having a diameter of 200 nm caused perforation of cell membranes [12]. Meanwhile, a study in which CNTs with a diameter of 15 nm were implanted in vivo showed that CNTs were taken up by macrophages but did not cause oncogenesis or severe inflammation [28, 29]. The NC7000 CNTs with a diameter of 9.5 nm used in the present study appeared to have less of an impact on the cells than 50 nm CNTs. However, further studies involving long-term in vivo observation are necessary to clarify the changes over time in CNT and CR adhesion. In addition, the physical and biological characteristics of CNTs that become separated from the resin surface on cell physiology also need to be examined.

Conclusion
In the present study, we coated the surface of composite resin with CNTs and investigated the biological response. CNT-CR surfaces enhanced cell proliferation compared with CR surface.

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